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Clostridium difficile in ready-to-eat foods in Isfahan and Shahrekord, Iran

Ebrahim Rahimi[1*], Zahra Sadat Afzali[2], Zeinab Torki Baghbadorani[3]

[1]*Department of Food Hygiene, College of Veterinary Medicine, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran*

[2]*Department of Biology, College of Basic Science, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran*

[3]*Department of Food Science and Technology, College of Agriculture, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran*

PEER REVIEW

Peer reviewer

Dr. Reza Sharafati Chaleshtori, Research Center for Biochemistry and Nutrition in Metabolic Diseases, Kashan University of Medical Sciences, Kashan, Iran. E-mail: psrpesar2@yahoo.com
Co-reviewer:
Prof. Haydar Özpinar, İstanbul Aydın University–34295, İstanbul, Turkey. Tel: 90 212 444 1 428/30001, Fax: 425 57 59, E-mail: haydarozpinar@aydin.edu.tr

Comments

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ABSTRACT

Objective: To determine the prevalence and antimicrobial resistance of *Clostridium difficile* (*C. difficile*) isolated from ready-to-eat foods of Iran.
Methods: From January to August 2013, a total of 368 unpacked ready-to-eat food samples were purchased from randomly selected supermarkets, retail stores and restaurants located in Isfahan and Shahrekord, Iran and were evaluated for the presence of *C. difficile*.
Results: *C. difficile* spores were detected in 5 (1.36%) of the 368 samples. The highest prevalence of *C. difficile* was found in fasl salad (4.29%), followed by yogurt stew (2%), and olovyeh salad (0.93%). All 140 maccaroni salad and falafel sandwich samples were negative for *C. difficile*. One of the five *C. difficile* isolates (20%) contained *tcdA*, *tcdB* and *cdtB* toxin genes and four strains (80%) contained *tcdA*, and *tcdB* toxin genes. Also, among the five *C. difficile* isolates, only three strains were found to be toxigenic for toxin A and/or B by ELISA. Isolates were susceptible to vancomycin and metronidazole, but variably resistant to other antimicrobial drugs.
Conclusions: This study, combined with studies on other food sources, suggests that widespread contamination of food is common.

KEYWORDS
Clostridium difficile, Ready-to-eat foods, ELISA

1. Introduction

Clostridium difficile (*C. difficile*)-associated diarrhea is a very common nosocomial infection. *C. difficile* is now the most commonly diagnosed cause of antimicrobial-associated and hospital-associated diarrhea, and the cause of virtually all cases of pseudomembranous colitis[1,2]. *C. difficile* has also been shown to be an important pathogen causing diarrhea in humans in communities outside hospital environments[3]. Incidence and severity of the disease appear to be increasing[4]. Moreover, *C. difficile* is also an important causative agent of diarrhea in immonosuppresed patients[5]. *C. difficile* infection has also been described in non-hospitalized patients without underlying disease or a predisposing risk factor[2,6]. *C. difficile* strains associated with the two conditions produce either toxin A (an enterotoxin) or toxin B (a cytotoxin), or both. Some strains do not produce toxins and do not cause diarrhea or pseudomembranous colitis.

C. difficile also appears to be an important cause of enteric disease in a wide variety of animal species[7-10], suggesting that animals and humans may share a common source for *C. difficile*

*Corresponding author: Ebrahim Rahimi, Department of Food Hygiene, College of Veterinary Medicine, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran.
Tel: +98 381 3361060
Fax: +98 381 3361060
E-mail: ebrahimrahimi55@yahoo.com
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infection[11,12]. Recent reports also show a remarkable overlap between isolates from animals and humans[13]. In other studies, *C. difficile* has been isolated from variety of food animals and foods of animal origin[9,12,14,15].

Possible community sources for *C. difficile* infection include soil, water, animals, meats and vegetables[2,16]. The mechanisms of transmission of *C. difficile* between animals and humans are not yet fully determined[2]. If animals are a potential source of *C. difficile*, food could be one of the transmission routes from animals to humans. Though there is currently no clear evidence that *C. difficile* contamination of food can cause *C. difficile* infection in humans, it is important to collect information on the possible exposure of humans to *C. difficile*-contaminated food. The aim of this study was to determine the occurrence of *C. difficile* in retail vegetable and ready-to-eat foods in Isfahan and Shahrekord, Iran.

2. Materials and methods

2.1. Sample collection

From January to August 2013, a total of 368 unpacked ready-to-eat food samples including fasl salad (n=70), macaroni salad (n=70), falafel sandwich (n=70), olovyeh salad (n=108) and yogurt stew (n=50) were purchased from randomly selected supermarkets, retail stores and restaurants located in Isfahan and Shahrekord, Iran. All samples were placed in separate sterile plastic bags to prevent spilling and cross contamination, and then immediately transported to the laboratory in a cooler with ice packs and processed within 6 h.

2.2. Isolation and identification of C. difficile

Food samples were analyzed for the presence of *C. difficile* using selective enrichment and isolation protocol described by Rodriguez-Palacios *et al*.[17] and Harvey *et al*[9]. About 5 g of a sample was aseptically transferred to 20 mL of *C. difficile* broth containing *C. difficile* selective supplement (Oxoid SR0173) and 5% (v/v) defibrinated sheep blood. After incubation at 37 °C for 10 to 15 d under anaerobic conditions, 2 mL of the enrichment was added into 2 mL of 96% ethanol in a centrifuge tube and homogenized for 50 min on a shaker. After centrifugation (3 800 r/min for 10 min), a loopful of material from the sediment was streaked onto *C. difficile* agar base (Oxoid CM0601) supplemented with an antibiotic supplement for the selective isolation of *C. difficile* (Oxoid SR0173), and 7% (v/v) defibrinated sheep blood. Then the plates were incubated for 48 h at 37 °C, under anaerobic conditions. Three colonies per plate were subcultured onto tryptone soya agar (Oxoid CM0131) and tested by standard microbiological and biochemical procedures[9]. Crudely extracted DNA (boiling for 10 min) was used for PCR confirmation (housekeeping *tpi* gene detection), and determination of toxin gene (*tcdA, tcdB* and *cdtB*) of isolates as performed in previous studies[15].

2.3. Determination of toxins A or B production

The isolates confirmed as *C. difficile* were cultured on sheep blood agar under anaerobic conditions at 37 °C for 2 d. After culturing, a thick bacterial cell suspension was prepared in 1 mL universal stool buffer (RIDASCREEN, R-Biopharm AG, Darmstadt, Germany) and centrifuged at 3000 r/min for 10 min. The supernatants were tested for the presence of *C. difficile* toxins A or B by ELISA detection kit (RIDASCREEN, R-Biopharm AG, Darmstadt, Germany) according to the manufacturer's instructions. Positive and negative controls were included in each batch.

2.4. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the Kirby–Bauer disc diffusion method using Mueller–Hinton agar (HiMedia Laboratories, Mumbai, India) according to the Clinical Laboratory Standards Institute standards previously described[7,9,18]. The antimicrobial agents tested and their corresponding concentrations were as follows: nalidixic acid (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), tetracycline (30 μg), doxycycline (30 μg), gentamicin (10 μg), metronidazol (5 μg), ampicillin (10 μg), chloramphenicol (30 μg), vancomycin (30 μg), and clindamycin (2 μg). After incubating the inoculated plate for 48 h at 37 °C, under anaerobic conditions, the susceptibility of *C. difficile* to each antimicrobial agent was measured and the results were interpreted in accordance with interpretive criteria provided by Clinical Laboratory Standards Institute[18].

2.5. Statistical analysis

Data were transferred to Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA) for analysis. Using SPSS 20.0 statistical software (SPSS Inc., Chicago, IL, USA), *Chi*-square test and Fisher's exact two-tailed test analysis were performed and differences were considered significant at values of P<0.05.

3. Results

Table 1 shows the prevalence of *C. difficile* isolated from ready-to-eat foods in Iran. *C. difficile* spores were detected in 5 (1.36%) of the 368 samples after culturing in enrichment broth (Table 1). The highest prevalence of *C. difficile* was found in fasl salad (4.29%), followed by yogurt stew (2.00%), and olovyeh salad (0.93%). All 140 maccaroni salad and falafel sandwich samples were negative for *C. difficile*. There were no significant differences (P>0.05) in the frequency of positive samples among the ready-to-eat food samples. Also, no significant differences in the prevalence rates were observed between vegetable samples isolated in the two provinces.

Table 1

Prevalence of *C. difficile* detected in ready-to-eat foods in Iran.

Food sample	No. of samples	No. of *C. difficile*-positive samples	No. of isolates positive for toxins			No. of isolates positive for toxins A and/or B
			tcdA	*tcdB*	*cdtB*	
Fasl salad[a]	70	3 (4.29%)	3	3	1	3
Maccaroni salad[b]	70	0 (0.00%)	-	-	-	-
Falafel sandwich[c]	70	0 (0.00%)	-	-	-	-
Olovyeh salad[d]	108	1 (0.93%)	-	-	-	-
Yogurt stew[e]	50	1 (2.00%)	1	1	-	1
Total	368	5 (1.36%)	4	4	1	4

[a]: Mostly contain lettuce, corn, mushroom, cabbage, onion and different spices. [b]: Mostly contain macaroni, pickle cucumber, corn, carrot and different spices. [c]: A special Iranian product prepared by frying the mixture of chickpea, onion, black pepper and served with tomato and pickle cucumber. [d]: Olovyeh is a mayonnaises based salad which mostly contain cooked chicken meat, potato, sour cucumber, green bean, salt and spices. [e]: Yogurt stew is a desert mostly served after dinner and it contain meat, egg yolk, saffron, sugar and yogurt.

One of the five *C. difficile* isolates (20%) was positive for *tcdA*, *tcdB* and *cdtB* toxin genes and four strains (80%) were positive for *tcdA*, and *tcdB* toxin genes (Table 1). Also, among five *C. difficile* isolates, four strains were found to be toxigenic for toxin A and/or B by ELISA.

The resistance of *C. difficile* isolates to 11 antimicrobial agents tested in this study is shown in Table 2. Resistance of *C. difficile* to clindomycin, nalidixic acid and gentamycin was high.

Table 2
Antimicrobial resistance of five *C. difficile* isolated from ready-to-eat foods in Iran.

Antimicrobial agent	Susceptible	Intermediate	Resistant
Ampicillin	2 (40%)	2 (40%)	1 (20%)
Chloramphenicol	4 (80%)	1 (20%)	0 (0%)
Ciprofloxacin	0 (0%)	1 (20%)	4 (80%)
Clindamycin	0 (0%)	0 (0%)	5 (100%)
Doxycycline	5 (100%)	0 (0%)	0 (0%)
Erythromycin	1 (20%)	2 (40%)	2 (40%)
Gentamicin	0 (0%)	1 (20%)	4 (80%)
Metronidazole	5 (100%)	0 (0%)	0 (0%)
Nalidixic acid	0 (0%)	0 (0%)	5 (100%)
Tetracycline	2 (40%)	1 (20%)	2 (40%)
Vancomycin	5 (100%)	0 (0%)	0 (0%)

4. Discussion

The present study determined the prevalence of *C. difficile* in ready-to-eat foods in Iran and showed that how foods may be the reservoirs of this organism. *C. difficile* were detected in 1.36% of the samples. Similarly, in a recent Scottish study, Bakri *et al*. found 3 (7.5%) of the 40 ready-to-eat salads samples positive for the presence of *C. difficile* spores by PCR[19]. In addition, a large study on *C. difficile* in South Wales reported isolation of *C. difficile* from 7 of 300 (2.3%) vegetables: two potatoes, one onion, one mushroom, one carrot, one radish, and one cucumber[20]. In a recent study in Iran, toxigenic *C. difficile* were detected from 2/7 (28.5%) hamburger processing plants, in 5.6% (3/54) of beef meat samples, 3.5% (2/56) of swabs taken from the environment and 7.1% (4/56) of hamburger samples after both molding and freezing[21]. In a study reported from Iran by Rahimi *et al*.[22], 13 of 660 meat samples (2%) including buffalo, beef, cow, sheep, and goat meat were contaminated with *C. difficile*. The highest prevalence of *C. difficile* was found in buffalo meat (9%). In another study conducted in Isfahan, Chaharmahal va Bakhtiari, and Khuzestan province of Iran, *C. difficile* was identified in 1.43% of 135 bulk milk samples [23].

Ribotype 027, and ribotype 078 are toxinotype III strain that has genes encoding *TcdA*, *TcdB* and *cdt*[2]. Likely, all of the *C. difficile* isolated in this study positive for *tcdA*, *tcdB* and *cdtB* toxin genes (n=1) were classified as ribotype 078 or 027. There is correspondingly little information about the types of *C. difficile* found in foodstuff especially vegetable and ready-to-eat foods. Although 71% of isolates from vegetables of South Wales were toxigenic[20], typing was not reported. All three isolates from the Scottish ready-to-eat salads were toxigenic, with two isolates being classified as ribotype 017 and one as ribotype 001[19]. Ribotype 017, ribotype 001, ribotype 078 and ribotype 027 are important in human disease. Contamination of food with these ribotype suggests a possible human health concern. Ribotype 078 and ribotype 027 has been reported to be the predominant type in food animals such as cattle and pigs[10,11], and other food sources[2,21].

Spores of *C. difficile* were found on food samples in different studies, but with very different prevalence rates between countries. However, it is not clear that whether this study reflects a true prevalence or the different prevalence between this study and other studies is due to that differences in sampling techniques employed, seasonal effects or laboratory methodologies employed in different studies are not clear[22].

The source of *C. difficile* in food products is unclear. There are various possible sources of vegetable contamination, such as soil, fertilizer (manure), water, processing environments, and human hands[2]. Also, the prolonged survival of *C. difficile* spores in the environment increases the possibilities of contamination in foods and infection in animals.

All the *C. difficile* isolates in this study were susceptible to doxycycline, metronidazol, and vancomycin as observed in other studies[17,23,24]. Metronidazol, and vancomycin are the most commonly used to treat *C. difficile* associated diarrhea. Our study demonstrates that there is resistance of *C. difficile* to clindomycin, gentamycin, and nalidixic acid, and other antimicrobials in our institution are comparable to those antibiotics as reported by other investigators[24,25]. The results of antimicrobial resistance founded in this study are correlated to antibiotics that are being used to treat infection in food animals in Iran.

According to the author's knowledge, the present study is the first report of the isolation of *C. difficile* from ready-to-eat foods in Iran. This study shows the importance of salad and vegetable as a potential source of transmission of *C. difficile* to humans, particularly since they are not cooked before being consumed. The consumption of these foods by vulnerable groups could possibly lead to *C. difficile* colonization and an increase in the asymptomatic *C. difficile* carriage rate among humans, thus increasing the risk for *C. difficile* transference within the healthcare environment[19]. Further studies are required to determine the potential risk of human infection with *C. difficile* via consumption of foods.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

C. difficile-associated diarrhea is a very common nosocomial infection and the cause of virtually all cases of pseudomembranous colitis. In recent studies *C. difficile* has been isolated from variety of food animals and foods of animal origin. Therefore it is required to determine the occurrence of *C. difficile* in retail vegetable and ready-to eat foods and other foods.

Research frontiers

The present research food samples were analyzed for the presence of *C. difficile* using selective enrichment medium and determined toxin A/B by ELISA. Also, PCR tests were used for determination of toxin gene (*tcdA*, *tcdB* and *cdtB*) of isolates. Finally, antimicrobial susceptibility testing was performed by disc diffusion method.

Related reports

Several studies reported isolation of *C. difficile* from vegetables as two potatoes, one onion, one mushroom, one carrot, one radish, and

one cucumber and ready-to-eat salads with very different prevalence rates between countries.

Innovations and breakthroughs

The present study is the first report of the isolation of *C. difficile* from ready-to-eat foods in Iran.

Applications

This study provides novel information for further studies to determine the potential risk of human infection with *C. difficile* via consumption of foods.

Peer review

This is a valuable research work in which authors have demonstrated the importance of salad and vegetable as a potential source of transmission of *C. difficile* to humans, particularly since they are not cooked before being consumed.

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